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Report No [redacted]

[redacted]

LOCAL LYMPH NODE ASSAY IN MICE (LLNA/IMDS)

[redacted]

Performing Laboratory:

[redacted]

Sponsor:

[redacted]

Study Completion Date: December 7, 2006

[redacted] [redacted]

GLP COMPLIANCE STATEMENT

This study was conducted in compliance with the OECD Principles of Good Laboratory Practice as revised in 1997 (ENV/MC/CHEM(98)17) and with the revised German Principles of Good Laboratory Practice according to Annex I German Chemicals Act (Bundesgesetzblatt Part I, No. 40 issued June 27, 2002).

[redacted]

(Study Director)

Nov. 10, 2006

Date

[redacted] [redacted]

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Quality Assurance Statement

Test Item : [redacted]

Study No.:

In the relevant laboratory areas the equipment and standard processes used in connection with toxicological short-term studies were audited periodically by the Quality Assurance (process- based inspections). Audit reports have been submitted in writing to the study director and to the laboratory management.

Date of Inspection	**Study phases inspected**	**Date of report to study director and/or management**
Oct. 19, 2006	STUDY PLAN	Oct. 19, 2006
Nov. 13, 2006	PREPARATION OF FORMULATION/FEEDMIXTURE, MEASUREMENTS, ADMINISTRATION, RAW DATA (PROCESS BASED)	Nov. 13, 2006
Nov. 29, 2006 - Nov. 29, 2006	FIRST DRAFT	Nov. 29, 2006
Dec. 05, 2006	FINAL DRAFT	Dec. 05, 2006

The results of the study and the methods used have been correctly reported.

Quality Assurance Unit

[redacted]

Date: Dec 05, 2006 **Responsible:** [redacted]

[redacted] [redacted]

2 SIGNATURES

Study Director: [redacted] Dec 7, 2006
Date

Head of Molecular and Special Toxicology: [redacted] Dec 7, 2006
Date

3 SUMMARY

The modified Local Lymph Node Assay (IMDS) was performed in 2006 on 24 female NMRI mice of the strain Hsd Win:NMRI (6 animals/test item group and 6 control animals) to determine if there is any specific (sensitizing) or non-specific (irritating) stimulating potential of the test item [redacted].

The study was conducted according to OECD Guideline No. 429, No. 406, EC Guideline 96/54/EC (22nd Adaptation of Guideline 67/548/EEC)/Health Effects Test Guideline, OPPTS 870.2600 (EPA) with the following test item concentrations:

0 (vehicle control), 1, 3 and 10%.

The test item was formulated in Acetone/Olive Oil (4:1) (A/OO) to yield a solution.

The results show that the test item ([redacted]) has a sensitizing potential in mice after dermal application. Compared to vehicle treated animals there was a clear increase in weights of the draining lymph nodes and in the cell counts at all dose groups. These changes are of statistical significance. The "positive level" of index 1.4 [6, 8, 9] was exceeded for the cell counts in all dose groups.

The "positive level" of ear swelling which is $2x10^{-2}$ mm increase [8, 9], i.e. about 10% of the control values, has been exceeded in the mid and high dose group (Appendix 11.2).
A significant increase compared to vehicle treated animals regarding ear swelling and ear weights was detected in the mid and high dose group.
An increase in this parameter would point to an acute irritating (inflammatory) response. However, such an irritating property could also be combined with a strong skin sensitizing potential of a test compound.

In conclusion, these results show that the test item ([redacted]) has a sensitizing potential in mice after dermal application.

[redacted] [redacted]

4 INTRODUCTION

A modified Local Lymph Node Assay (IMDS) was carried out in mice with the following test item:

[redacted]

The modifications refer to the measurement of cell proliferation by cell counting instead of radioactive labeling. In addition, the acute inflammatory skin reaction is determined to discriminate specific from non-specific activation of immune competent cells in the draining lymph nodes [cf. 4, 6, 8, 9, 10, 23-25].

The aim of these investigations was to establish whether there is any specific (sensitizing) or non-specific (irritating) stimulating potential of the test item [redacted]

The investigations were carried out at the [redacted] in [redacted]

The study plan, raw data and the final report are retained in the archives specified by [redacted].
The storage of a retention sample of the test item and, if applicable, also of the reference item is in the responsibility of the sponsor.

[redacted] [redacted]

5 STUDY IDENTIFICATION AND RESPONSIBILITIES

5.1 Study identification

The laboratory study was carried out under number [redacted]

Study number: [redacted]

Experimental starting date: October 23, 2006
Experimental completion date: October 26, 2006
Study completion date: see signature page

Sponsor: [redacted]

5.2 Responsibilities

Head of HealthCare Toxicology:

Head of Molecular and Special Toxicology:

Study Director:

Head of Quality Assurance Unit:

Archiving of study data:

[redacted]

6 MATERIAL AND METHODS

The methods used in this study are in principle specified in guidelines (OECD 406, 1992; EPA guideline OPPTS 870.2600, Skin Sensitization, March 2003; CPMP/SWP/2145/00, 2001; OECD 429, 2002). According to these guidelines the so-called Local Lymph Node Assay (LLNA) is recommended for the assessment of skin sensitization as first-stage screening study or as a stand-alone test (OECD 429; OPPTS 870.2600).

The principle of the method had been published in 1989 [1], and a first collaborative validation study in 1991 [2]. In these first trials the stimulation of the lymph nodes, i.e. cell proliferation, was measured by ^{3}H-Thymidin incorporation. In 1999 the principle of the LLNA had been stated as valid alternative to guinea pig assays by the ICCVAM [3], although the need for further modifications was also noted.

A modification of the assay by measuring the cell counts instead of radioactive labeling provides comparable sensitivity [4, 5], and has the advantage that the cell suspension can be further analyzed by different methods (flow cytometry, chemiluminescence responses, immunofluorescence) to gain an insight into mechanistic events [4-7]. A further modification was done by including the measurement of the ear swelling after treatment leading to a much more simplified and reliable assay (Integrated Model for the Differentiation of Skin reactions (IMDS), [8]). By comparing the specific immune reaction induced by the test item in the draining lymph nodes (LN; cell counts / LN weights) with the immediate unspecific acute skin reaction (ear swelling / ear weight) it is possible to discriminate the irritating potential from the sensitizing potential of the compound tested. International standards have successfully determined using this modification [9, 24, 25]. Such modifications are also authorized in the Note of Guidance SWP/2145/00 of the CPMP (2001) and OECD guideline 429.
With respect to this simple discrimination between sensitizing and irritating local reactions comparable findings have been reported in the human patch test system [10].

[redacted]

6.1 Test item

Test item:	[redacted]
Chemical name:	[redacted]
Content*:	100%
Batch No.:	[redacted]
Molecular mass:	[redacted]
Approval:	until January 27, 2007
Physical state:	liquid
Appearance:	clear, brownish
Storage*:	room temperature

* based on the product information given by the sponsor

6.2 Animals

6.2.1. Selection of the species

SPF-bred female NMRI mice of the strain Hsd Win:NMRI were used from commercial breeder [redacted]. Animals of this strain have been used for years for toxicity studies at [redacted]. Mice of this strain have also been used for the intra-laboratory validation of the IMDS [8, 9], as well as an inter-laboratory validation study [24, 25].
Historical data on their physiology and spontaneous alterations are available. The health status of the strains is checked regularly at random for the most important specific infection pathogens, and the results of these tests are stored at [redacted]

[redacted]

6.2.2. Adaptation

After their arrival, the animals intended for the study were allowed to adapt to the conditions of the animal room for at least 7 days and their state of health was monitored.

6.2.3. Health status

Only healthy animals showing no signs of disease were used in the study. The animals were not vaccinated or treated with antiinfectives either before their arrival or during the adaptation or study period. The females were nulliparous and nonpregnant.

6.2.4. Age and Body weight

The mice exhibited a weight of 26 – 32 grams at the beginning of the study. The age of the animals was 9 weeks.

6.3 Housing of the animals

6.3.1. Housing conditions

During the adaptation period up to 8 mice were housed together in conventional Makrolon® type III cages [11, 12], while during the study period the animals were single-housed in type II cages. The cages were changed at least twice a week.

Low-dust wood granulate from J. Rettenmaier & Söhne Füllstoff-Fabriken, 73494 Rosenberg, Germany, was used as bedding. At the instigation of the Laboratory Animal Services (LAS) the wood granulate was analyzed at random for contaminants. The relevant documents have been retained. The analytical results have not yielded evidence of any influence on the study objective.

6.3.2. Animal rooms

All the animals used in this study were kept in the same room. At times animals taking part in other toxicological studies were kept in the same room, but adequate spatial separation and appropriate organization of the working procedures ensured that animals could not be confused.

[redacted] [redacted]

6.3.3. Cleaning, disinfection, and pest control

The animal room was cleaned once a week and disinfected at least once a month with Terralin (20 g benzalkonium chloride and 35 g Phenoxypropanol/100 g; diluted to 1% for use, i.e. 10 ml in 1 litre of water). Contamination of the feed and contact with the animals were excluded.

Pest control was not carried out in the animal room, but Killgerm Roach Traps which use no pesticides were placed in the animal room for cockroach control.

6.3.4. Environmental conditions

The environmental conditions in the animal room were standardized as follows:

Room temperature:	22 ± 2° C
Relative humidity:	40%-70%
Light/dark cycle:	12 h/12 h, with artificial illumination
Air throughput:	About 10 changes per hour

Occasional deviations from these conditions occurred e.g. as a result of the cleaning of the animal room, but they had no apparent effect on the course of the study.

6.3.5. Diet

The feed, PROVIMI KLIBA SA 3883 maintenance diet for rats and mice (from Provimi Kliba SA, CH-4303 Kaiseraugst), and tap water (drinking bottles) were provided ad libitum.

The nutritive composition of PROVIMI KLIBA SA 3883 and the contaminant content of the standard diet was checked and analyzed routinely in random samples. The tap water was of drinking water quality [13].

The results of the feed and water analyses have been stored at [redacted]. The available data do not show evidence of any effects on the study objective.

Polycarbonate bottles with a capacity of about 300 ml (study period) or 700 ml (adaptation period) were used for drinking water [11, 12].

6.4 Methods

The method used has been described in the literature (see above). Unless internationally recognized standardized reference values or tests are available, the method used here must be viewed with this in mind. On the other hand there are enough peer reviewed data available confirming the validity of this method [reviewed in 3, 4-9].

6.4.1. Methodological Reliability

The Local Lymph Node Assay Test methodology was checked for reliability in a test on female NMRI mice using Alpha Hexyl Cinnamic Aldehyde formulated in different vehicles (PEG 400, DAE 433, DMF, MEK, Aceton/Olive Oil (4:1) and Cremophor EL/ physiological saline solution 2% v/v) at concentrations of 3%, 10% and 30%.
The sensitivity as well as the reliability of the experimental technique is thus confirmed by this study [26].
A similar check is done in regular intervals using one of the above mentioned vehicles in order to confirm the reliability of the method [27].

The last reliability test using Alpha Hexyl Cinnamic Aldehyde formulated in Acetone/Olive Oil (4:1) at concentrations of 3%, 10% and 30% showed clearly the sensitizing potential of the test item.

6.4.2. Procedure

6.4.2.1. Grouping and identification of the animals

Six animals were placed in each group.
The animals were identified by cage labels giving the test item, the animal number, dose, sex, and the study number and marking of the tail immediately before autopsy.

6.4.2.2. Test item formulation

The test item was formulated once at day 1 of the study in A/OO.
The formulations were visually described as solutions.
The stability of the test item in the vehicle was analytically verified for up to 4 days.

[redacted] [redacted]

6.4.2.3. Results of the Stability of the test item in the formulation

Nominal value mg/ml	Content mg/ml			Content as % of nominal value		
	start	after 2 hours	after 4 days	start	after 2 hours	after 4 days
9.77	9.80	9.77	9.80	100	100	100
505.0	475.5	502.8	493.6	94	100	98

Data taken from Study number: [redacted]

6.4.2.4. Route of administration and dosage

The test item (described in Section 6.1) in the formulation (described in Section 6.4.2.2) or the vehicle were applied epicutaneously onto the dorsal part of both ears of the animals. This treatment was repeated on three consecutive days (d1, d2 and d3).

The volume administered was 25µl/ear.

Based on our experiences with this test system and the toxic properties of the test item the following concentrations were used: 0 (vehicle control), 1, 3 and 10%.

6.4.3. Investigations

6.4.3.1. Autopsies

The animals were anaesthetized by inhalation of carbon dioxide and sacrificed one day after the last application (day 4). The appropriate organs were then removed. Lymphatic organs (the auricular lymph nodes) were transferred into physiological saline (PBS).

6.4.3.2. LLN Weight and cell count determinations

The weight and cell count determinations were carried out by appropriate laboratory procedures. The weights of the lymph nodes were determined on a Mettler semiautomatic balance and stored in a IBM compatible PC. After crushing the lymph nodes through a sieve in a 12-well plate, the cell counts per ml were determined using a Multisizer 3® from Coulter Electronics. These data were also directly collected and processed by computer (Multisizer 3 software and Excel). Means, indices and standard deviations were calculated by an Excel data sheet.
A special BASIC program (GWBASIC compiler) was used to calculate means and standard deviations of the Lymphnodes' weights. Indices were calculated manually.

The so-called stimulation (or LLN-) index is calculated by dividing the absolute number of weight or cell counts of the substance treated lymph nodes by the vehicle treated ones. Thus, in case of no stimulating effect the index is always about 1.00 (+/- standard deviation), and the indices of vehicle treated animals are set to 1.00 (+/- standard deviation).
The samples (cell suspensions) of this study have been analyzed by flow cytometry (FACScan) in addition. These analyzes are not part of the GLP-study (routine lymph node assay (IMDS/LLNA)). The results serve to collect more data of the different subpopulations involved in skin reactions.

6.4.3.3. Ear Swelling

Before the first treatment and before sacrifice the thickness of both auricles of the animals was measured using a spring-loaded micrometer (Oditest, Dyer Company). Means, indices and standard deviations of the ear swelling were calculated by an Excel data sheet.

6.4.3.4. Ear weight

On day 4 of the study the ear weight of the sacrificed animals was measured using a punch to take of a piece of every ear with a diameter of 8 mm. The weights were determined on a Mettler semiautomatic balance. Means, indices and standard deviations of the ear weights were calculated by an Excel data sheet.

6.4.3.5. Body weights

The body weights of the animals were recorded at the start and the end of the study (day 1 and day 4); cf. Appendix 11.3.

[redacted] [redacted]

6.4.4. Statistics

When it was statistically reasonable, the values from treated groups were compared with those from the control group(s; vehicle) by a one-way analysis of variance (ANOVA) [15, 16] when the variances are considered homogeneous according to a homogeneity testing like Cochran's test [17]. Alternatively, if the variances are considered to be heterogenous ($p \leq 0.05$), a non-parametric Kruskal-Wallis test has been used (Kruskal-Wallis ANOVA) at significance levels of 5%. Two sided multiple test procedures were done according to Dunnett [18, 19] or Bonferroni-Holm [20], respectively. Outlying values in the LN weights were eliminated at a probability level of 99% by Nalimov's method [21]. In addition, for the LLNA/IMDS the smallest significant differences in the means were calculated by Scheffe's method [22], which according to Sachs [17] can be used for both equal and unequal sample sizes.
In this method of statistical processing of measurements a large number of comparisons is made, and as a result of the multiple tests the overall probability of error is considerably greater than the p values suggest (increased number of false-positive results). On the other hand, the known methods of adjusting p values lead to an excessive increase in the number of false negatives. In view of these problems the biological and toxicological relevance is also taken into consideration in the evaluation of statistical significance.

For this reason, in the case of indices only the standard deviations between groups and difference analysis of the mean values were used in the evaluation of the biological relevance.

7 RESULTS

7.1 Stimulation indices (weight and cell counts; ear swelling and ear weight)

Based on results obtained in validation studies and general experiences with this test system (see Section 6 and 6.4) groups of mice were treated with vehicle, 1, 3 or 10% [redacted] in A/OO.

All dose groups (1, 3 and 10%) of the NMRI mice showed a clear increase in the weights of the draining lymph nodes and in the stimulation indices for cell counts (Appendix 11.1 and 11.2, 1.) compared to control animals after application of the test item [redacted] The "positive level" which is 1.4 for cell count indices has been exceeded in all dose groups. These increases are of statistical significance.

The "positive level" of ear swelling which is $2x10^{-2}$ mm increase [8, 9], i.e. about 10% of the control values, has been exceeded in the mid and high dose group (Appendix 11.2, 2.). A statistical significant increase of the ear weights (Appendix 11.2, 3.) and ear swelling compared to control animals was also detected for the mid and high dose group.

It has to be clarified that the "positive levels" mentioned above are exclusively defined for the NMRI outbred mice used for this study [8, 9]. Such positive limits have to be calculated for each strain of mice individually [24, 25].

7.2 Body weights

The body weights of the animals were not affected by any treatment (Appendix 11.3).

8 DISCUSSION AND EVALUATION

A LLNA/IMDS was carried out in female NMRI mice after epicutaneous application of a formulation containing 0, 1, 3 or 10% of the test item [redacted] for 3 consecutive days onto both ears of the animals.

The study indicate that the LLNA/IMDS does point to a specific immunostimulating (skin sensitizing) potential of the test item.

This applies to NMRI mice, for weight and cell counts of the draining lymph nodes as well as ear swelling and ear weight indices evaluated after application of [redacted]

After treatment with [redacted] there was a clear increase compared to control animals regarding the weights of the draining lymph nodes and the stimulation indices for cell counts of all dose groups. The "positive level" which is 1.4 for cell counts has been exceeded in all dose groups. These increases are of statistical significance.

A sensitizing potential can be assumed from the increases in cell proliferation in the draining lymph nodes. On the basis of our experiences using this method the "positive level" had been set to an increase in cell count index by 0.4 (i.e. index $\geq$ 1.4), which has been exceeded in all dose groups. Differentiation indices (DI) calculated according to our publications [8, 9] which is the quotient of the relative lymph node reaction divided by the relative acute skin reaction were > 1 for all concentrations tested, i. e. 5.42, 5.01 and 3.31, respectively. These DI values do also point to a skin sensitizing potential of the test item.

The "positive level" of ear swelling which is $2x10^{-2}$ mm increase [8, 9] has been exceeded in the mid and high dose group (Appendix 11.2). An increase in this parameter would point to an acute irritating (inflammatory) response. However, such an irritating property could also be combined with a strong skin sensitizing potential of a test compound.

Taken together, a specific activation of the cells of the immune system via dermal route was determined after application of 1, 3 and 10% [redacted] by the method used. Thus, [redacted] has to be classified as a skin sensitizer.

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Toxicology **212** (2005), 69-79

26. [redacted]

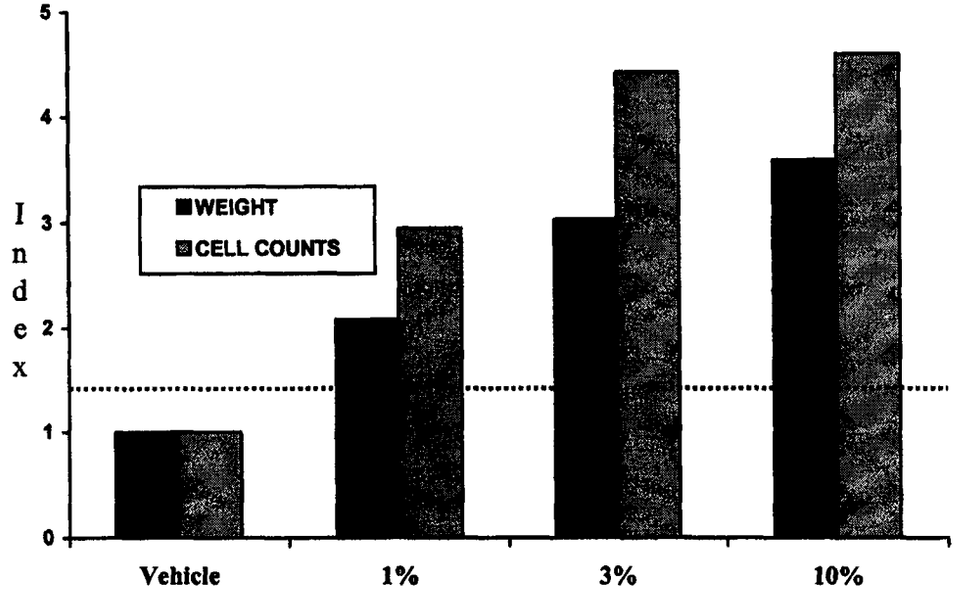
27. [redacted]

10 ABBREVIATIONS

A/OO	acetone/olive oil (4:1)
B	B cell
CC	cell counts
^{o}C	degrees centigrade
cf	confer
d	day
DAE 433	dimethylacetamide (40%), acetone (30%) and ethanol (30%)
DMF	dimethylformamide
DMSO	dimethylsulfoxide
FACScan	Fluorescence Activated Cell Scanner
h	hour
IMDS	Integrated Model for the Differentiation of chemical-induced Skin reactions
kg	kilogram
LN	lymph node
LLN	local lymph node
LLNA	local lymph node assay
MEK	methyl ethyl ketone
mg	milligram
ml	milliliter
MO	macrophage
NaCl	sodium chloride
PBS	phosphate buffered saline
PEG 400	polyethylene glycol 400
Pluronic/ NaCl	Pluronic PE 9200/ 0.9% NaCl solution, 1% v/v
rel.	relative
SD	standard deviation
T	T cell
Veh.	vehicle
v/v	volume/volume
w/w	weight/weight

11 APPENDIX

11.1 Bar charts (weight and cell count) for the LLNA



11.2 Tabular summary of the LLNA/IMDS results

1. Direct LLNA (NMRI mice, female, 6 animals/group)

Dose (%)	**Weight index** (**mean** +/- SD in %)	**Cell count index** (**mean** +/- SD in %)
0*	**1.00** +/- 22.90	**1.00** +/- 32.79
1	**2.08**↑ +/- 21.73	**2.95**↑ +/- 30.21
3	**3.03**↑ +/- 19.09	**4.41**↑ +/- 32.30
10	**3.59**↑ +/- 12.45	**4.58**↑ +/- 13.51

2. Ear swelling (NMRI mice, female, 6 animals/group, in 0.01 mm)

Dose (%)	day 1 (**mean** +/- SD in %)	day 4 (**mean** +/- SD in %)	Index day 4
0*	**17.08** +/- 3.01	**17.25** +/- 3.60	1.00
1	**17.58** +/- 3.80	**18.75** +/- 4.02	1.09
3	**17.42** +/- 5.17	**20.17**↑ +/- 5.92	1.17
10	**17.25** +/- 2.62	**21.92**↑ +/- 6.29	1.27

* = A/OO
↑ = statistically significant increase ($p \leq 0.05$)

[redacted] [redacted]

3. Ear weight (NMRI mice, female, 6 animals/group, in mg per 8 mm diameter punch)

Dose (%)	day 4 (**mean** +/- SD in %)	Index day 4
0*	**10.79** +/- 6.64	1.00
1	**11.38** +/- 8.27	1.05
3	**12.36**↑ +/- 8.55	1.15
10	**13.58**↑ +/- 4.21	1.26

* = A/OO
↑ = statistically significant increase ($p \leq 0.05$)

[redacted]

11.3 Body weights

Animal-No.	Body weight in g	
	Day 1	**Day 4**
group 1 control		
1	30	30
2	29	29
3	28	28
4	28	28
5	27	27
6	26	27
Mean	**28.0**	**28.2**
group 2 1%		
7	28	30
8	31	32
9	32	34
10	31	33
11	30	30
12	28	28
Mean	**30.0**	**31.2**
group 3 3%		
13	29	30
14	32	34
15	28	27
16	31	33
17	28	29
18	30	28
Mean	**29.7**	**30.2**
group 4 10%		
19	30	28
20	28	27
21	30	30
22	28	28
23	27	31
24	31	28
Mean	**29.0**	**28.7**